

Anti-inflammatory Effects of Amentoflavone on Modulation of Signal Pathways in LPS-stimulated RAW264.7 Cells

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Amentoflavone is naturally occurring bioflavonoid that is found in a number of plants. In this paper, the anti-inflammatory activity of amentoflavone in LPS-stimulated macrophages and its mode of action were examined. Using LPS-stimulated RAW264.7 macrophage cells, we found that amentoflavone exerted anti-inflammatory activities through inhibition of nitric oxide (NO) production and tumor necrosis factor (TNF)- α and macrophage inflammatory protein (MIP)-2 secretion. Amentoflavone (1.0-20 μ M) gradually inhibited nitrite production without cytotoxicity. Amentoflavone (1.0 and 10 μ M) effectively suppressed both TNF- α and MIP-2 cytokine release from LPS-stimulated RAW264.7 cells. The expression of mL-1 β and mMIP-2 cytokine mRNAs was completely inhibited while expression of mMIP-1 was effectively suppressed and mTNF- α expression was slightly inhibited by 10 μ M amentoflavone. We also demonstrated that the innate immune response to amentoflavone involves the toll-like receptor (TLR) and mitogen-activated protein kinase (MAPK) pathways. LPS-induced upregulation of p38 MAPK phosphorylation was significantly reduced by 10 μ M amentoflavone. These results suggest that amentoflavone exhibits effective anti-inflammatory activities through regulation of TLR4 and phosphorylation of p38 MAPKs.

Key Words : Flavonoid, Amentoflavone, Anti-inflammatory activity, Cytokine, TLR4

Introduction

Inflammation is a protective biological response to harmful stimuli, pathogens, or irritants in vascular tissues that attempts to eliminate infectious stimuli.¹ Wounds would never be cured without inflammation. However, chronic inflammation can cause diseases, namely, hay fever, atherosclerosis, and rheumatoid arthritis. That is why inflammation is closely monitored and physically regulated. Nitric oxide (NO) is an inflammatory mediator that is associated with inflammation and immune regulation.^{2,3} Inflammatory cytokines are produced when transcriptional regulators such as mitogen-activated protein kinases (MAPKs) are activated.^{4,5} The MAPKs, especially p38 MAPK and extracellular signal-regulated kinase (ERK), are regarded as the essential regulators of pro-inflammatory molecules in the cellular responses that occur following induction of inflammatory gene transcription.^{6,7} Flavonoids are widely distributed polyphenolic compounds with antioxidative, anti-inflammatory, and anti-tumor activities.⁸ Flavonoids have been shown to exhibit biological effects in various diseases, including antihepatotoxic, antiatherogenic, antiallergic and anticancer activities, as well as anti-inflammatory activity.⁹ Among the flavonoids, flavone, the isoflavones daidzein and genistein, the flavanols isorhamnetin, kaempferol and quercetin, the flavanone naringenin, and the anthocyanin pelargonidin inhibited LPS-stimulated NO production and iNOS expression in macrophages. Genistein, kaempferol, quercetin, and daidzein suppressed transcription factors of STAT-1 and NF- κ B, whereas

flavone, isorhamnetin, naringenin, and pelargonidin exclusively controlled NF- κ B.¹⁰ Flavonoids compete with ATP binding and eventually inhibit tyrosine kinases and serine kinases.¹¹⁻¹³ Novogrodsky and coworkers showed that tyrosine kinase blocking agents like tyrphostins inhibit LPS-stimulated TNF- α production.¹⁴

Among the numerous flavonoids, amentoflavone is found in a variety of plants with pharmacological properties such as *Ginkgo biloba* and *Hypericum perforatum*.¹⁵ Amentoflavone exerts anti-bacterial, anti-inflammatory and antioxidative activities.¹⁶⁻²⁰ The production of nitric oxide, nitric oxide synthase (iNOS), and NF- κ B was suppressed by amentoflavone in LPS-stimulated macrophages. Furthermore, amentoflavone inhibited the activation of group II phospholipase A₂ and cyclooxygenase which are known to be involved in inflammatory conditions.^{18,21} Amentoflavone is a bioflavonoid that is bis-apigenin coupled at the 8 and 3' positions. It has been reported that amentoflavone has inhibitory activity against group II phospholipase A₂ on plates from synovial fluids.²² Secreted phospholipase A₂ is known to induce inflammation in mammalian cells. High levels of phospholipase A₂ expression cause inflammatory diseases and promote the vascular inflammation related to coronary artery disease.²³ In our previous study, we also verified that amentoflavone exhibited strong anticancer activities against MCF-7 and HeLa cancer cells through activation of hPPAR γ , which is regulated by PTEN activation.²⁴ Cancer often appears to occur in sites of infection, chronic irritation and inflammation. Various kinds of cyto-

kines and chemokines are produced in tumor cells.²⁵ It has been reported that amentoflavone effectively inhibited NO production in LPS-stimulated RAW264.7 cells.²⁶ However, the anti-inflammatory signal transduction of amentoflavone has not been reported, yet. Therefore, in this study, we investigated the anti-inflammatory activities of amentoflavone and its mechanism of action. We hypothesized that mRNA expression of inflammatory cytokines such as TNF- α , iNOS, MIP-1, MIP-2, and IL-6 will be increased in LPS-stimulated cells, and that this regulation is dependent on the TLR4 and p38 pathways. We investigated these hypotheses using RT-PCR and western blotting. Herein, we report that amentoflavone exhibits anti-inflammatory activity and inhibits production of LPS-stimulated pro-inflammatory cytokines, and we systemically determined our current understanding of the mechanisms underlying activated TLR4 and MAPKs regulation.

Methods

Extraction and Isolation of Amentoflavone. A whole *Selaginella tamariscina* plant (600 g) was extracted with methanol at room temperature, yielding 50.54 g of residue. The methanol extract was resuspended in water and sequentially partitioned with dichloromethane, ethyl acetate, and *n*-butanol. The ethyl acetate fraction (3.0 g) was placed on a silica gel column (300 g, 4.8 \times 45 cm) and eluted using a chloroform/methanol/H₂O gradient (120:10:1, 80:10:1, 50:10:1, 20:10:1, 10:10:1, and 0:10:0, v/v). Based on their TLC pattern, the fractions were combined to yield sub-fractions, which were designated E1–10. Subfraction E7 (296.33 mg) was purified by repeated column chromatography over a silica gel, RP-18, and Sephadex LH 20, yielding amentoflavone (82.23 mg). The UV, ¹H NMR, and ¹³C NMR data for amentoflavone were identical to those reported.²⁷

Cytotoxicity Against RAW264.7 Cells. The mouse macrophage-derived RAW264.7 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotic solution (100 units/mL penicillin and 100 μ g/mL streptomycin) at 37 °C in a humidified 5% CO₂ atmosphere. The cytotoxicity of amentoflavone against RAW264.7 was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.²⁸

Quantification of Nitrite Production in LPS-stimulated RAW264.7 Cells. Nitrite accumulation in culture media was used as an indicator of NO production.²⁹ RAW264.7 cells were plated at a density of 1 \times 10⁵ cells/mL in 96-well culture plates and stimulated with 20 ng/mL of lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma-aldrich, MO, USA) in the presence or absence of amentoflavone for 24 h. Isolated supernatant fractions were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at rt for 10 min. Nitrite

production was determined by measuring absorbance at 540 nm, and converted to nitrite concentrations by reference to a standard curve generated with NaNO₂.

Quantification of Inflammatory Cytokines (mTNF- α and mMIP-2) in LPS-stimulated RAW264.7 Cells. Antibodies against mouse tumor necrosis factor- α (mTNF- α) and mouse macrophage inflammatory protein-2 (mMIP-2) were immobilized on immunoplates by incubation with 0.2–0.8 μ g/mL solutions of antibody in PBS overnight at room temperature (rt). Plates were washed once with PBS/0.1% Tween-20 (PBST) and blocked by incubation with 200 μ L of blocking solution (3% bovine serum albumen [BSA] and 0.02% NaN₃ in PBS) overnight at rt. Then, the supernatants from LPS-stimulated RAW264.7 cells co-incubated with serially diluted amentoflavone for 18 h were added to the wells of the pre-coated plates and incubated for 2 h at rt. After washing plates with PBST, biotinylated-anti-mTNF- α and biotinylated-anti-mMIP-2 antibodies (0.4 μ g/mL) were added, and the plates were incubated for 2 h. After washing, the plates were incubated with streptavidin peroxidase (0.3 μ g/mL). SureBlue TMB peroxidase substrate (KPL, Inc., Gaithersburg, MD, USA) was added and the reaction was allowed to proceed at rt for color development. Finally, color development was stopped by adding 100 μ L of 1 M H₂SO₄. The absorbance at 450 nm was measured using a microplate reader. All values represent the means \pm standard deviations of at least 3 independent experiments.³⁰

Reverse Transcription-polymerase Chain Reaction. RAW264.7 cells were plated in 6-well plates at 5 \times 10⁵ cells/well and cultured overnight. Cells were stimulated with 100 ng/mL LPS or unstimulated (negative control) in the presence or absence of amentoflavone in RPMI-1640 supplemented with 1% penicillin/streptomycin for 3 h. Competitive reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously.³¹ Briefly, total RNA was extracted using an RNeasy kit (QIAGEN, Hilden, Germany) and total RNA was reverse transcribed into cDNA using oligo(dT)15 primers. The targets were amplified from the resulting cDNA by PCR using the following specific primers: interleukin-1 β (IL-1 β), 5'-CTG TCC TGA TGA GAG CAT CC-3' (sense) and 5'-TGT CCA TTG AGG TGG AGA GC-3' (antisense); macrophage inflammatory protein (MIP)-1, 5'-ATG AAG CTC TGC GTG TCT GC-3' (sense) and 5'-TGA GGA GCA AGG ACG CTT CT-3' (antisense); MIP-2, 5'-ACA CTT CAG CCT AGC GCC AT-3' (sense) and 5'-CAG GTC AGT TAG CCT TGC CT-3' (antisense); TNF- α , 5'-GTT CTG TCC CTT TCA CTC ACT G-3' (sense) and 5'-GGT AGA GAA TGG ATG AAC ACC-3' (antisense); iNOS, 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3' (sense) and 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3' (antisense). The primers for glyceraldehyde 3-phosphate (GAPDH), used as an internal control, were 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (antisense). PCR was performed using the following cycling conditions: 94 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1.5 min, and 94 °C for 1 min, with a final extension

step at 72 °C for 5 min. Amplified products were electrophoresed on 1% agarose gels, and bands were visualized by UV illumination of ethidium bromide-stained gels.

Western Blotting. RAW264.7 cells were seeded in 6-well plates at 3×10^6 cells/well and incubated in RPMI-1640 (Welgene, Daegu, Korea) supplemented with 10% FBS (Invitrogen, NY, USA) and antibiotics (100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin; Invitrogen) at 37 °C under 5% CO_2 . Cultured cells were stimulated with 100 ng/mL LPS for 6 h, after which the cells were incubated overnight with amentoflavone (10 μM). After incubation, cells were washed twice with PBS and detached using ice-cold PBS. The collected cells were centrifuged at 1,000 rpm for 5 min at 4 °C. Cell pellets were resuspended in 100 μL of lysis buffer (1% Triton X-100, 1% deoxycholate, and 0.1% NaN_3) and incubated for 30 min on ice. Lysed cells were centrifuged at 12,000 rpm for 10 min at 4 °C, and the concentration of protein in the supernatant (cytoplasmic extract) was determined using a Bradford assay (Bio-Rad, Hemel Hempstead, UK). Equal amounts of protein (20 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked by incubation with 5% skim milk in Tris Buffered Saline with Tween 20 (TBST; 25 mM Tris, 3 mM KCl, 140 mM NaCl, and 0.1% Tween 20) for 1 h at rt, and then incubated with antibodies specific for TLR 4 (1:1,000; Cell Signaling Technology, Beverly, MA, USA), phospho-p38 MAPK (1:1,000; Cell Signaling Technology), and β -actin (1:5,000; Sigma-Aldrich, St. Louis, MO, USA). After washing with TBST buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit Immunoglobulin G (IgG) or anti-mouse IgG (1:10,000; Sigma-Aldrich) secondary antibodies, as appropriate. Signals were detected using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Buckinghamshire, UK).

Results and Discussion

Cytotoxicity of Amentoflavone in RAW264.7 Cells. To

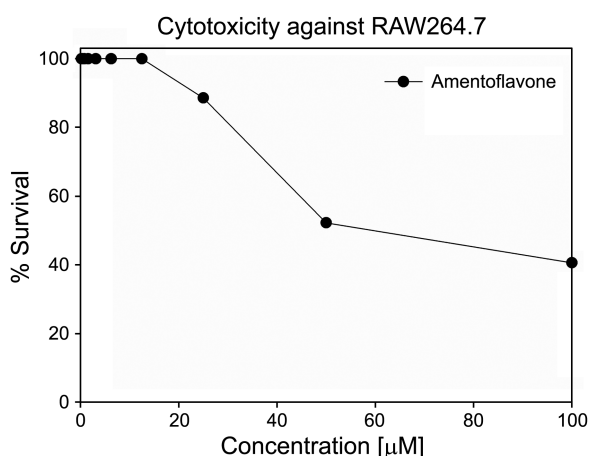


Figure 1. Dose-response curve for the cytotoxicity of amentoflavone in macrophage-derived RAW264.7 cells.

find a concentration of amentoflavone that is not toxic to RAW264.7 macrophages, we investigated cytotoxicity using the MTT assay in RAW264.7 mouse macrophage cells. As shown in Figure 1, at 25 mM amentoflavone, the survival rate of RAW264.7 cells was greater than 89%, while the survival rates in 50 mM and 100 mM amentoflavone were 52% and 41%, respectively.

Quantification of Nitrite Production in LPS-Stimulated RAW264.7 Cells. To demonstrate the anti-inflammatory activity of amentoflavone, we directly measured amentoflavone-mediated inhibition of NO production in LPS-stimulated macrophage RAW264.7 cells using the quantifiable nitrite method. We investigated the inhibition of NO production at various concentrations (1 μM , 2.5 μM , 5 μM , 10 μM , and 20 μM). As shown in Figure 2(a), amentoflavone at 1.0–20.0 μM gradually inhibited NO production in LPS-stimulated RAW264.7 macrophages. Amentoflavone at 5 μM , 10 μM , and 20 μM inhibited NO production by 20%, 38%, and 59%, respectively compared to NO production in non-treated cells. Up to 5 μM amentoflavone exhibited anti-inflammatory activities and was non-toxic to RAW264.7 cells. Therefore, these data showed that amentoflavone is non-toxic at concentrations that have anti-inflammatory activity.

Quantification of Inflammatory Cytokines (mTNF- α and mMIP-2) in LPS-stimulated RAW264.7 Cells. Since NO production was significantly inhibited by amentoflavone, we then measured the mRNA expression of inflammatory-induced cytokines in LPS-stimulated RAW264.7 macrophage cells. The inflammation-induced cytokines we examined

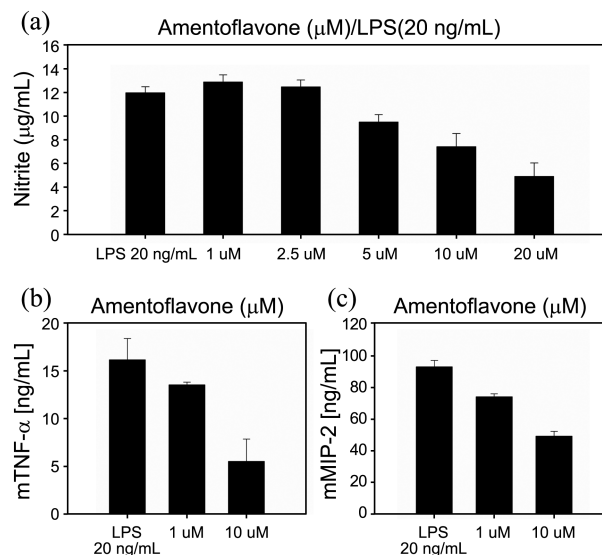


Figure 2. (a) Inhibition of NO production by amentoflavone (1, 2.5, 5, 10, and 20 μM) in LPS-stimulated RAW264.7 cells. The error bar represents the standard deviation for 3 independent experiments. (b) Inhibition of mTNF- α production by 1 and 10 μM amentoflavone in LPS-stimulated RAW264.7 cells. The error bar represents the standard deviation for 3 independent experiments. (c) Inhibition of mMIP-2 production by 1 and 10 μM amentoflavone in LPS-stimulated RAW264.7 cells. The error bar represents the standard deviation for 3 independent experiments.

were mTNF- α and mMIP-2, and the concentrations of mTNF- α and mMIP-2 were directly measured. A quantitative analysis of mTNF- α and mMIP-2 demonstrated that amentoflavone clearly inhibited both mTNF- α and mMIP-2 production in LPS-stimulated mouse macrophage cells. In amentoflavone-treated (1 μ M and 10 μ M) macrophage cells mTNF- α cytokine levels decreased 17% and 66%, respectively, compared with that in non-treated cells, while mMIP-2 cytokine levels decreased 20% and 47%, respectively (Figure 2(b) and (c)).

Reverse Transcription-Polymerase Chain Reaction. We investigated whether amentoflavone regulated mRNA expression of inflammatory cytokines. When 100 ng/mL of LPS was added to RAW264.7 cells, the expression of inflammatory cytokines (mTNF- α , miNOS, mMIP-1, mMIP-2, and mIL-1 β) was detected in macrophages by RT-PCR. iNOS is the enzyme responsible for the generation of NO, and it is an important enzyme that regulates inflammatory responses. As shown in Figure 3, the expression of mIL-1 β and mMIP-2 mRNA was inhibited 86% and 100%, respectively, in amentoflavone-treated (10 μ M) cells compared to the mRNA levels in non-treated cells. The expression of mTNF- α and mMIP-1 mRNA was suppressed 24% and 48%, respectively, in amentoflavone-treated (10 μ M) cells compared with the mRNA levels in non-treated cells. Therefore, amentoflavone

(10 μ M) effectively suppressed the expression of mIL-1 β and mMIP-2 mRNA and slightly inhibited the expression of mTNF- α and mMIP-1 mRNA.

Western Blotting. To determine the mechanism of NO production inhibition, we examined the effect of amentoflavone on TLR4 and MAPKs activation. Three types of MAPKs are expressed, including ERKs, c-Jun NH2-terminal kinases, and p38 MAPKs. Recent studies showed that the MAPKs were activated by LPS. RAW264.7 cells were treated with amentoflavone with or without LPS, and western blotting was performed to detect TLR4 and phospho-p38. As

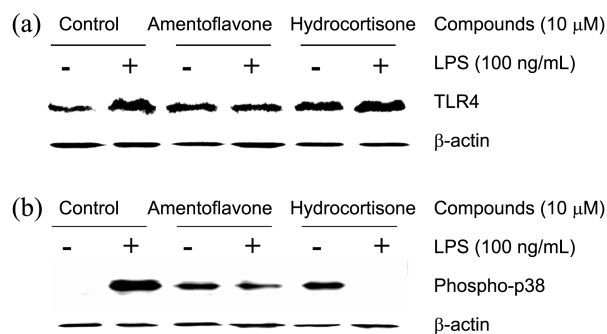


Figure 4. Western blots showing the concentration-dependent effects of amentoflavone on the expression of TLR4 and phospho-p38 in LPS-stimulated RAW264.7 cells.

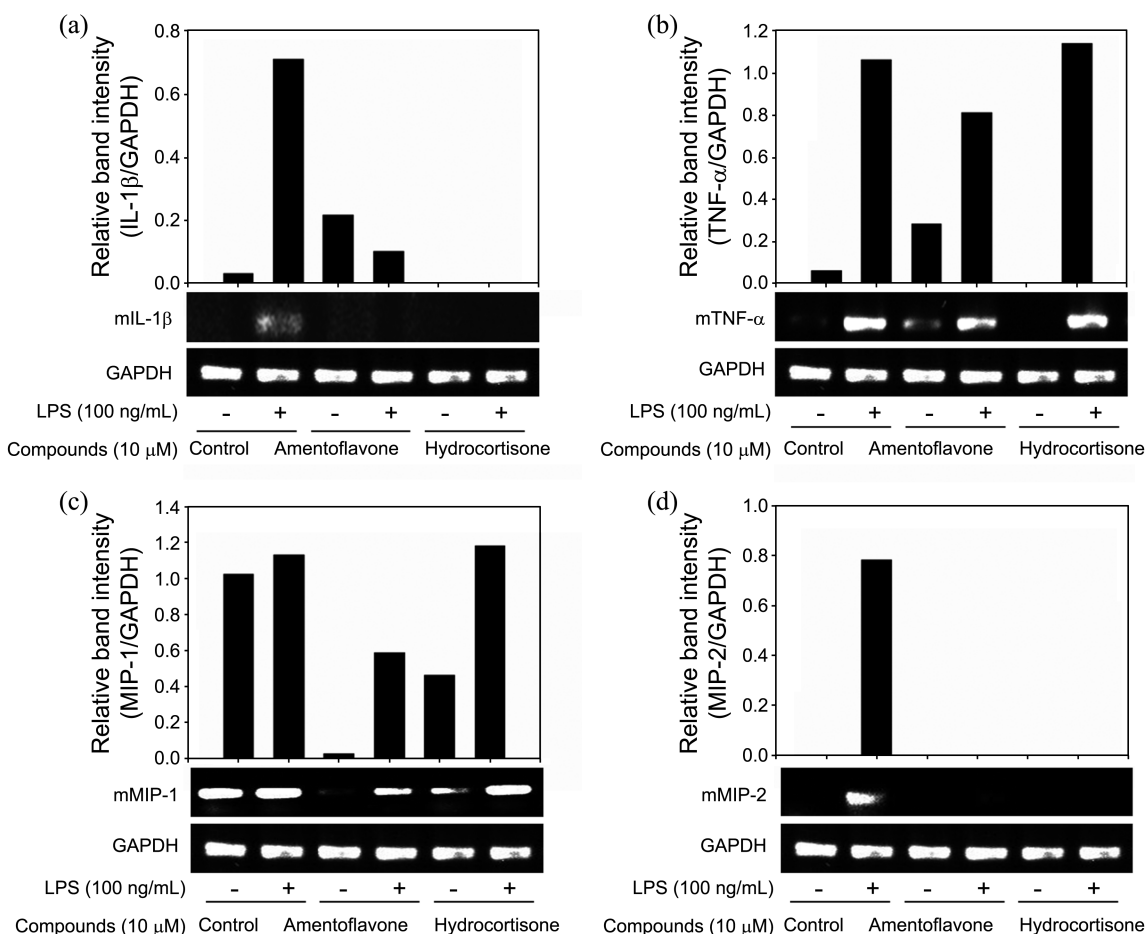


Figure 3. Effect of amentoflavone on inflammation-induced cytokine mRNA expression in LPS-stimulated RAW264.7 cells. GAPDH was used as a control. The relative mRNA expression was quantified using image J (NIH, Bethesda, MD, USA).

shown in Figure 4, amentoflavone regulated LPS-induced phosphorylation of TLR4 and phosph-p38 compared with hydrocortisone. Hydrocortisone is the corticosteroids class of drugs, used to reduce inflammatory disease. The LPS-induced expression of TLR4 and p38 was significantly reduced by 10 mM amentoflavone treatment. Therefore, amentoflavone was shown to inhibit NO production by decreasing TLR4 and p38 expression.

Conclusion

In this study, we demonstrated that amentoflavone at non-toxic concentrations significantly inhibited NO production from LPS-stimulated macrophages. As the concentration of amentoflavone increased, less NO was produced. We investigated how the mRNA expression of several cytokines is related to NO production. We measured the expression of TNF- α , mMIP-1, mMIP-2, and IL-1 β mRNA by RT-PCR. TNF- α is known to induce neutrophil proliferation during inflammatory events. MIP-1 and MIP-2 are chemotactic cytokines that are related to the immune response during infection and inflammation, and IL-1 β is known to be an important mediator of the inflammatory response. Therefore, these cytokines have pivotal functions in inflammation. Among the cytokines tested, the expression of mL-1 β and mMIP-2 were strongly inhibited in LPS-stimulated macrophages, while mTNF- α and mMIP-1 were considerably suppressed in macrophages treated with 100 ng/mL LPS and 10 μ M amentoflavone. In addition to RNA expression, we also tested TLR and MAPKs expression by western blotting. Amentoflavone regulated the expression of TLR4 and the phosphorylation of p38 in LPS-stimulated macrophage cells. Figure 4 shows that LPS-stimulated phosphorylation of p38 was significantly reduced by amentoflavone treatment. Phosphorylation of p38 is upregulated by LPS treatment alone and is downregulated by treatment with LPS and amentoflavone. Accordingly, the p38 MAPKs pathway might be the downstream signaling pathway of TLR4. Therefore, these might be suitable targets for inflammatory disease therapeutics. The mechanism underlying the anti-inflammatory action of amentoflavone will be further investigated.

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